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14. ABSTRACT

The focus of this grant is on development of a novel model system for propagation and quantitation of mammalian prions: the budding yeast *Saccharomyces cerevisiae*. This unicellular organism offers a number of potential advantages for the study of prion biology, including rapid generation time, ease of culturing, and facile genetics. There is a strong conservation of cellular mechanisms between yeast and mammalian cells, particularly as regards the biogenesis, trafficking and localization of membrane proteins. Thus, although yeast do not express an endogenous PrP-like molecule, there are strong reasons to believe that they possess the molecular machinery to allow propagation of mammalian prions. We hypothesize that the only additional requirement is the provision of a source of membrane-anchored PrP^{C}, the essential substrate for conversion into PrP^{Sc}. Using *S. cerevisiae* strains that express engineered forms of PrP^{C}, we propose to: (1) Determine whether mammalian prions can be propagated in yeast; (2) Develop methods for titering prions in yeast; (3) Characterize the phenotype of prion-infected yeast; and (4) Identify genes that modulate prion propagation in yeast.

During years 2 and 3 of the grant, we initiated a new line of investigation that makes use of PrP-expressing yeast (Task 5). This new project is aimed at investigating an intriguing and potentially important hypothesis concerning the normal, physiological function of PrP^{C}.

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INTRODUCTION

The focus of this grant is on development of a novel model system for propagation and quantitation of mammalian prions: the budding yeast *Saccharomyces cerevisiae*. This unicellular organism offers a number of potential advantages for the study of prion biology, including rapid generation time, ease of culturing, and facile genetics. There is a strong conservation of cellular mechanisms between yeast and mammalian cells, particularly as regards the biogenesis, trafficking and localization of membrane proteins. Thus, although yeast do not express an endogenous PrP-like molecule, there are strong reasons to believe that they possess the molecular machinery to allow propagation of mammalian prions. We hypothesize that the only additional requirement is the provision of a source of membrane-anchored PrP^C, the essential substrate for conversion into PrP^{Sc}. Using *S. cerevisiae* strains that express engineered forms of PrP^C, we propose to: (1) Determine whether mammalian prions can be propagated in yeast; (2) Develop methods for titering prions in yeast; (3) Characterize the phenotype of prion-infected yeast; and (4) Identify genes that modulate prion propagation in yeast.

During years 2 and 3 of the grant, we initiated a new line of investigation that makes use of PrP-expressing yeast (Task 5). This new project is aimed at investigating an intriguing and potentially important hypothesis concerning the normal, physiological function of PrP^C.

BODY

Task 1: Determine whether mammalian prions can be propagated in yeast (months 1-12).

a. Infect yeast using three different protocols.

b. Test for prion propagation using biochemical and bioassay methods.

We have carried out additional experiments in which we incubated spheroplasts with either purified PrP^{Sc} or liposomes incorporating PrP^{Sc}. Bulk or clonal cultures were then assayed for PrP^{Sc} by Western blotting after protease treatment. Thus far, we have not been able to detect amplification of PrP^{Sc} in infected cultures.

Our initial set of yeast strains expressed PrP from an ADH-driven, 2 micron plasmid. We had initially planned to repeat the infection experiments using strains in which PrP is expressed using a GPD-driven promoter. However, we found that PrP expression levels are similar using the ADH and GPD promoters, so this approach is unlikely to represent an improvement. Also, given the lack of detectable PrP 27-30 in inoculated cultures, we do not think it worthwhile to perform bioassays in mice or hamsters to detect low levels of infectious prions, particularly given the residual inoculum that is likely to be present in the cultures.

Given the negative results obtained thus far, we do not feel that it is worthwhile pursuing further infection experiments (Tasks 1-4). We have therefore initiated a new line of investigation with PrP-expressing yeast (Task 5) that has yielded impressive results related to elucidation of the normal, physiological function of PrP^C.

<u>Task 2</u>: Develop methods for titering prions in yeast (months 6-18).

Not begun.

<u>Task 3</u>: Characterize the phenotype of prion-infected yeast (months 12-24).

Not begun.

<u>Task 4</u>: Identify genes that modulate prion propagation in yeast (months 24-36).

Not begun.

<u>Task 5 (new)</u>: Use yeast to investigate a role for PrP^C in protection of cells from pro-apoptotic stress (months 18-36).

Several lines of evidence suggest that PrP^C, the non-infectious form of the prion protein, may function to protect neurons and other cells from stress or toxicity. We therefore investigated the use of *S. cerevisiae* as a model system to assay the cytoprotective activity of PrP^C. The mammalian pro-apoptotic protein, Bax, confers a lethal phenotype when expressed in yeast. Since over-expression of PrP^C has been found to prevent Bax-mediated cell death in cultured human neurons, we explored whether PrP could also suppress Bax-induced cell death in yeast. We utilized DPAPB-PrP, the form of mouse PrP containing a modified signal peptide that we had previously shown is efficiently targeted to the secretory pathway in yeast. We found that this PrP potently suppressed the death of yeast cells expressing mammalian Bax under control of a galactose-inducible promoter. In contrast, cytosolic PrP (23-231) failed to rescue growth of Bax-expressing yeast, indicating that protective activity requires targeting of PrP to the secretory pathway. Deletion of the octapeptide repeat region did not affect the rescuing activity of PrP, but deletion of a charged region encompassing residues 23-31 partially eliminated activity. We also tested several PrP mutants associated with human familial prion diseases, and found that only a mutant containing 9 extra octapeptide repeats failed to suppress Bax-induced cell death. These findings establish a simple and genetically tractable system for assaying a putative biological activity of PrP^C.

We have recently developed an improved method for assaying the Bax-suppressive activity of PrP in *S. cerevisiae.* Our initial assay involved spotting serial dilutions of yeast cultures on glucose or galactose plates, and then visually observing the amount of growth after several days. However, we found that this assay was difficult to quantitate, and offered little dynamic range to measure different degrees of Bax suppressive activity. After experimenting with several alternative methods for scoring yeast viability, we settled upon a colony-formation assay. Yeast are grown for 24-48 hrs in either glucose or galactose medium, and the number of viable cells is then scored by plating on glucose plates. This assay shows a strong killing effect of Bax, and a robust rescue by co-expression of PrP. Importantly, the PrP rescue effect is observed even when PrP expression is driven by the ADH promoter. The ADH promoter produces lower expression levels than the GPD promoter we used previously, thus eliminating a substantial PrP-induced toxicity observed even in the absence of Bax. Other advantages of the new assay are that it is quantitative and has a large dynamic range.

We are currently using the colony formation assay to test several new candidate genes to determine if they are essential for PrP suppression of Bax-induced cell death. These include Ire1, Sir2, and several genes involved in apoptotic pathways in mammalian cells.

We are currently developing the tools we need to carry out deletion and suppressor screens in yeast in order to identify additional genes required for PrP rescue activity. Our original idea of using absorbance values of yeast grown in glucose vs. galactose to score viability is not workable, since dead yeast cells make a substantial contribution to the absorbance values. We are currently focusing on the use of fluorescent vital dyes, so that the number of live yeast cells can be measured directly using a microtiter plate reader. With the use of appropriately designed genetic screens and selections, it should now be possible to identify proteins from yeast and mammals that alter the cytoprotective activity of PrP and that may also interact physically with PrP.

KEY RESEARCH ACCOMPLISHMENTS

- Development of methods for assaying the anti-apoptotic activity of PrP in yeast.
- Structure-function analysis of the anti-apoptotic activity of PrP in yeast.
- Analysis of the effect of disease-related mutations on the anti-apoptotic activity of PrP in yeast.

REPORTABLE OUTCOMES

Publication: Li, A., and Harris, D.A. (2005). Mammalian prion protein suppresses Bax-induced cell death in yeast. J. Biol. Chem. 280:17430-17434.

CONCLUSIONS

During year 2, we carried out further experiments attempting to infect PrP-expressing yeast with rodent prions. Given the negative results obtained, we elected to suspend work on Tasks 1-4. Instead, we initiated a new line of investigation (Task 5) aimed at elucidating the normal, physiological function of PrP^C. We have now established that PrP can efficiently rescue yeast from Bax-induced cell death, and have carried out an analysis of which regions of the protein are essential for this effect. We believe that this system now represents the simplest, most robust, and most genetically facile system for assaying the physiological activity of PrP^C. Large-scale screens carried out using this system are likely to identify other proteins that interact physically or genetically with PrP.

This new line of investigation is directly relevant to understanding the pathogenesis of prion diseases, since there is now considerable evidence that the normal biological activity of PrP^C plays an essential role in the toxicity of prions. Thus, loss or subversion of the anti-apoptotic activity of PrP^C is likely to be an essential part of the mechanism by which prions kill cells. Of course, understanding how prions produce their pathogenic effects is essential for the development of effective therapeutics and diagnostics. The new line of investigation we have developed during years 2 and 3 is therefore fully consistent with the original objectives of the grant program.

REFERENCES

Li, A., and Harris, D.A. (2005). Mammalian prion protein suppresses Bax-induced cell death in yeast. J. Biol. Chem. 280:17430-17434.

APPENDICES

Publication included as a pdf file.

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Mammalian Prion Protein Suppresses Bax-induced Cell Death in Yeast*Ⓢ

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Several lines of evidence suggest that PrP^C, the non-infectious form of the prion protein, may function to protect neurons and other cells from stress or toxicity. In this paper, we report on the use of the yeast *Saccharomyces cerevisiae* as a model system to assay the cytoprotective activity of PrP^C. The mammalian pro-apoptotic protein, Bax, confers a lethal phenotype when expressed in yeast. Since overexpression of PrP^C has been found to prevent Bax-mediated cell death in cultured human neurons, we explored whether PrP could also suppress Bax-induced cell death in yeast. We utilized a form of mouse PrP containing a modified signal peptide that we had previously shown is efficiently targeted to the secretory pathway in yeast. We found that this PrP potently suppressed the death of yeast cells expressing mammalian Bax under control of a galactose-inducible promoter. In contrast, cytosolic PrP-(23–231) failed to rescue growth of Bax-expressing yeast, indicating that protective activity requires targeting of PrP to the secretory pathway. Deletion of the octapeptide repeat region did not affect the rescuing activity of PrP, but deletion of a charged region encompassing residues 23–31 partially eliminated activity. We also tested several PrP mutants associated with human familial prion diseases and found that only a mutant containing nine extra octapeptide repeats failed to suppress Bax-induced cell death. These findings establish a simple and genetically tractable system for assaying a putative biological activity of PrP^C.

Prion diseases are a group of transmissible neurodegenerative disorders, including Creutzfeldt-Jakob disease, Gerstmann-Sträussler syndrome, kuru, and fatal familial insomnia in human beings, as well as scrapie and bovine spongiform encephalopathy in animals (1). These diseases are caused by the conversion of PrP^C,[1] a normal cell surface glycoprotein, into PrP^{Sc}, a β-sheet-rich conformer that is infectious in the absence of nucleic acid (2, 3). Although a great deal is known about the role of PrP^{Sc} in the disease process, the normal function of PrP^C has remained elusive. A variety of functions have been proposed for PrP^C, including roles in metal ion trafficking (4), cell adhesion (5), and transmembrane signaling (6). Identifying the function of PrP^C may provide important clues to the pathogenesis of prion diseases, since there is evidence that PrP^C plays an essential role in mediating the neurotoxic effects of PrP^{Sc} (7).

Several intriguing lines of evidence have emerged recently indicating that PrP^C may function to protect cells from various kinds of internal or environmental stress (8). For example, PrP overexpression rescues cultured neurons and some mammalian cell lines from pro-apoptotic stimuli, including Bax expression, serum withdrawal, and cytokine treatment (9–12). In addition, PrP-null mice are more susceptible to neuronal loss after experimental brain injury (13), and neurons cultured from these animals display abnormalities related to increased susceptibility to oxidative stress (14). Finally, expression of wild-type PrP completely abrogates the neurodegenerative phenotype of mice expressing the PrP paralogue, Doppel, or N-terminally truncated forms of PrP (Δ32–121 and Δ32–134) (15, 16).

In this paper, we have utilized the yeast *Saccharomyces cerevisiae* as a experimentally accessible model system in which to analyze the cytoprotective function of PrP^C. In particular, we tested whether PrP could rescue yeast from cell death induced by the pro-apoptotic protein, Bax, similar to the way PrP has been shown to protect cultured human neurons from Bax-induced death (9, 12). We took advantage of the fact that heterologous expression of mammalian Bax in yeast is lethal (17), a phenomenon that has been used as an assay to isolate Bax inhibitor proteins from mammalian sources (18–20). We also capitalized on our recent demonstration that PrP with a modified signal peptide is efficiently targeted to the yeast secretory pathway, where it becomes glycosylated, glycolipid-anchored, and localized to the plasma membrane as it does in mammalian cells (21). We show here that PrP targeted to the secretory pathway efficiently suppresses Bax-induced cell death in yeast, and we perform a structure-function analysis to determine which features of the PrP molecule are essential for this effect. Our results establish a simple and genetically tractable system for assaying a putative biological activity of PrP^C.

EXPERIMENTAL PROCEDURES

Yeast—Strain YPH499 (*MATa ura3–52 lys2–801 ade2–101 trp1-Δ63 his3-Δ200 leu2-Δ1*) was obtained from Stratagene (La Jolla, CA). Yeast were grown at 30 °C on SD (synthetic medium containing glucose as the sole carbon source) or SG (synthetic medium containing galactose as the sole carbon source), with drop-out of either Ura, Leu, or both.

Plasmids—Murine Bax was expressed using the vector pESC-LEU (Stratagene, La Jolla, CA), which contains a 2-micron origin of replication, two galactose-inducible promoters (GAL10 and GAL1), and a leucine (Leu) selection marker. The murine Bax coding sequence was amplified by PCR from a full-length cDNA clone (IMAGE 3968903; Open Biosystems, Huntsville, AL) using the following primers: 5′-TA-

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Ⓢ The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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[1] The abbreviations used are: PrP, prion protein; PrP^C, cellular isoform of PrP; PrP^{Sc}, scrapie isoform of PrP; BI-1, Bax inhibitor 1; DPAPB, dipeptidyl aminopeptidase B; GPD, glyceraldehyde-3-phosphate dehydrogenase; GPI, glycosylphosphatidylinositol; HA, hemagglutinin; SD, synthetic dextrose (glucose) medium; SG, synthetic galactose medium; WT, wild-type.

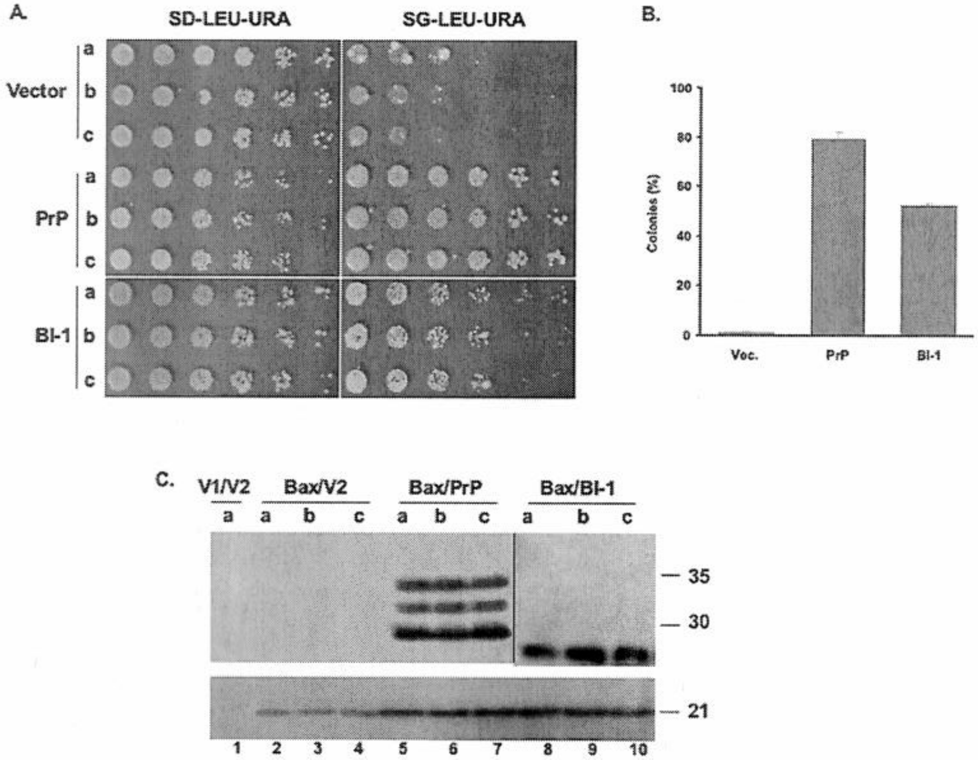

FIG. 1. **PrP rescues yeast from Bax-induced cell death.** *A*, yeast expressing Bax under control of a galactose-inducible promoter (in the vector pESC-LEU) were transformed with empty p426GPD vector (*Vector*) or with p426GPD vector encoding either DPAPB-PrP254 (*PrP*) or HA-tagged, murine BI-1 (*BI-1*). Cultures of three independent transformants (*a–c*) were grown overnight in SD-Leu-Ura liquid medium and then diluted to equal densities. 4-Fold serial dilutions of each culture were spotted (*left* to *right*) onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates and then grown for 3 or 6 days, respectively. *B*, yeast transformed as in *A* were spread at equal densities on SD-Leu-Ura or SG-Leu-Ura plates, and the number of colonies that grew after 3 or 6 days, respectively, was counted. Results are expressed as the number of colonies on the galactose plates as a percentage of those on the glucose plates. *Bars* represent the mean ± S.E. of counts of colonies from three independent transformants. *Vec.*, vector. *C*, cultures of transformants *a–c* (*A*) were grown to log phase in SD-Leu-Ura liquid medium and were then transferred to SG-Leu-Ura medium for 18 h to induce Bax expression. Equal amounts of protein extract were subjected to Western blotting using anti-PrP (*upper panel*, *lanes 1–7*), anti-HA (*upper panel*, *lanes 8–10*), or anti-Bax (*lower panel*, *lanes 1–10*) antibodies. *Lane 1* shows an extract from yeast carrying the empty vectors pESC-LEU (V1) and p426GPD (V2) used for expression of Bax and PrP/BI-1, respectively. Molecular size markers are in kilodaltons.

TAATTAAGCTTGCGGCCGCATGGACGGGTCCGGGGAG-3′ (forward) and 5′-CCGCGGTCTAGATCTTCAGCCCATCTTCTTCCAGATGGT-3′ (reverse). The forward primer includes a NotI restriction site, and the reverse primer includes a BglII restriction site. The NotI/BglII-digested PCR product was cloned into pESC-LEU that had been cut with the same two enzymes, which places the Bax coding region under control of the GAL10 promoter.

All PrP constructs were derived from the murine PrP sequence and contained the 3F4 epitope tag (22). All constructs were expressed using the vector p426GPD, which contains a 2-micron origin of replication, a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter, and a uracil (Ura) selection marker (23). The coding region of DPAPB-PrP254 was subcloned from pVT102U into p426GPD using BamHI and HindIII sites. The construction DPAPB-PrP254/pVT102U has been described previously (21).

Deletion and truncation mutants of PrP (Δ51–90, Δ32–80, Δ32–93, Δ23–31, 23–231, Δ231–254 (GPI$^-$)) were generated by bridge PCR using DPAPB-PrP254 as a template. PCR products were then cloned into p426GPD via BamHI and HindIII sites. Point and insertional mutants of PrP (PG14, E199K, P101L, D177N, F197S) were created by removing a KpnI/XbaI fragment from DPAPB-PrP254/pVT102U and substituting the equivalent fragment excised from previously constructed pcDNA3 plasmids carrying these mutations (24). The DPAPB-PrP254 coding regions were then subcloned from pVT102U into p426GPD via BamHI and HindIII sites.

The coding region of murine BI-1 (18) with a single C-terminal HA tag (YPYDVPDYA) was amplified by PCR from a full-length cDNA clone (IMAGE 3708463; Open Biosystems) and was cloned into p426GPD via BamHI and HindIII sites.

Yeast cells were transformed by electroporation (25).

Protein Analysis—Proteins were extracted from yeast by lysis in NaOH/β-mercaptoethanol as described previously (26) and were resuspended in SDS sample buffer. After heating for 10 min at 95 °C (PrP, Bax) or 56 °C (BI-1), proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. PrP was detected with monoclonal antibody 3F4 (22), Bax with rabbit anti-Bax antibody N-20 (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse BI-1 with anti-HA monoclonal antibody 12CA5 (Roche Diagnostics). Proteins were visualized using an ECL kit (Amersham Biosciences).

Proteinase K resistance and detergent insolubility were assayed as described previously (21).

RESULTS

PrP Rescues Yeast from Bax-induced Cell Death—We expressed murine Bax in *S. cerevisiae* using a galactose-inducible promoter (GAL10), so that growth in the presence or absence of Bax could be assessed by plating on galactose medium (SG) or glucose medium (SD), respectively. Overnight cultures were serially diluted and spotted on both kinds of plates. Yeast carrying the inducible Bax plasmid grew well on SD-Leu but failed to grow on SG-Leu (supplemental Fig. S1*A*). In contrast, yeast transformed with empty vector grew similarly on both plates. Western blotting confirmed that growth in galactose medium induced expression of Bax (supplemental Fig. S1*B*). Thus, inducible expression of Bax is lethal in yeast.

To test whether mammalian PrP could rescue yeast from Bax-induced cell death, we utilized DPAPB-PrP254, a form of PrP containing a chimeric signal peptide consisting of the signal-anchor sequence of the yeast protein DPAPB fused to the C-terminal 7 amino acids of the PrP signal peptide. We have shown previously that DPAPB-PrP254 is efficiently targeted to

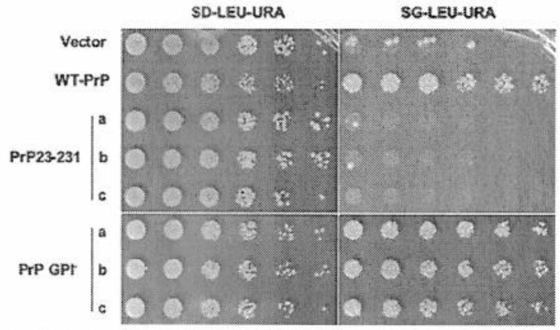

FIG. 2. **Cytosolic PrP does not suppress Bax-induced cell death, but non-membrane-anchored PrP does.** Yeast expressing Bax under control of a galactose-inducible promoter were transformed with empty p426GPD vector (*Vector*) or with p426GPD vector encoding DPAPB-PrP254 (*WT-PrP*), PrP23–231, or DPAPB-PrP230 (*PrP GPI⁻*). Serial dilutions of cultures of three independent transformants (*a–c*) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.

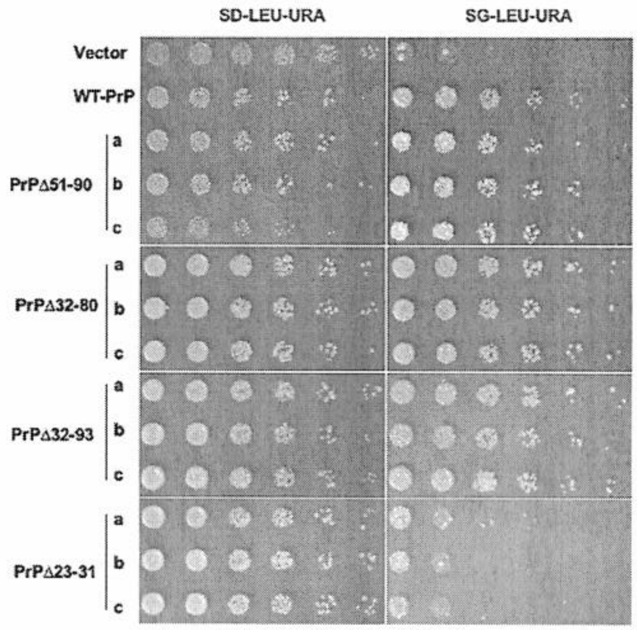

FIG. 3. **Deletion of residues 23–31, but not the octapeptide repeats, reduces rescue activity.** Yeast expressing Bax under control of a galactose-inducible promoter were transformed with empty p426GPD vector (*Vector*) or with p426GPD vector encoding DPAPB-PrP254 (*WT-PrP*) or DPAPB-PrP254 carrying the indicated deletions (Δ51–90, Δ32–80, Δ32–93, and Δ23–31). Serial dilutions of cultures of three independent transformants (*a–c*) were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1.

the secretory pathway in yeast, where it is glycosylated, GPI-anchored, and trafficked to the plasma membrane (21). Bax-expressing yeast were transformed with a plasmid that expresses DPAPB-PrP254 under control of the strong, constitutive GPD promoter. As controls, yeast were transformed with empty vector or with the same vector encoding murine Bax inhibitor 1 (BI-1), which has previously been shown to rescue yeast from Bax-induced cell death (18). Overnight cultures of three independent transformants of each strain were serially diluted and spotted on glucose (SD-Leu-Ura) and galactose (SG-Leu-Ura) plates.

Each of the strains grew on glucose plates, although yeast expressing DPAPB-PrP254 were slightly inhibited in their growth compared with the other two strains (Fig. 1*A*). On galactose plates, as expected, vector-transformed yeast failed to grow, while yeast expressing BI-1 were partially rescued. Remarkably, yeast expressing DPAPB-PrP254 grew even better on galactose than yeast expressing BI-1. Quantitation of the number of colonies on galactose plates compared with glucose plates revealed that DPAPB-PrP254 rescued 80% of the growth in the presence of Bax, compared with 50% for BI-1 and 2% for empty vector (Fig. 1*B*). We also noticed that PrP-expressing yeast produced larger colonies than BI-1-expressing yeast on galactose plates (Fig. 1*A*), implying that PrP is more potent than BI-1 in enhancing growth rate as well as increasing the number of surviving cells in the presence of Bax. Western blotting confirmed expression of Bax, DPAPB-PrP254, and BI-1 in yeast strains grown in galactose medium, demonstrating that DPAPB-PrP254 and BI-1 do not interfere with expression of Bax (Fig. 1*C*). The data shown in Fig. 1 were obtained using the yeast strain YPH499, but similar results were obtained with strain BY4741 (data not shown).

We performed two additional control experiments. To exclude the possibility that the expression of DPAPB-PrP254 enhanced growth on galactose medium independent of Bax, we analyzed yeast expressing only DPAPB-PrP254 without Bax. We found that expression of DPAPB-PrP254 did not enhance, and actually slightly inhibited, growth on SG-Ura, when comparison was made to yeast transformed with empty vector (supplemental Fig. S2*A*). To confirm that the growth rescuing effect was due to the presence of the DPAPB-PrP254 plasmid and not to another genetic alteration, we selected for yeast that had lost this Ura-marked plasmid by growth on 5-fluoroorotic acid. We found that yeast that had lost the plasmid did not retain the ability to grow on galactose medium (supplemental Fig. S2*B*).

Cytosolic PrP Does Not Suppress Bax-induced Cell Death, but Non-membrane-anchored PrP Does—To investigate the rescuing activity of cytosolic PrP, Bax-expressing yeast were transformed with a plasmid encoding PrP23–231. This protein lacks both the N-terminal signal peptide and the C-terminal GPI addition signal and would therefore be expected to be localized to the yeast cytosol. We found that yeast expressing PrP23–231 failed to grow on SG-Leu-Ura medium, similar to vector-transformed yeast, indicating that cytosolic PrP is incapable of rescuing yeast from Bax-induced cell death (Fig. 2). We did not note any difference in growth of the two strains on SD-Leu-Ura, demonstrating that cytosolic PrP is not toxic in the absence of Bax. Western blots confirmed that PrP23–231 was being expressed and that, in contrast to DPAPB-PrP254, it was not glycosylated, consistent with failure to enter the secretory pathway (supplemental Fig. S3*A*).

To test whether GPI anchoring to the cell membrane was essential for the rescuing activity of PrP, we tested yeast transformed with a plasmid encoding DPAPB-PrP230, which contains the chimeric signal sequence but is missing the C-terminal, GPI addition signal. This form of PrP displayed full rescue activity, as indicated by similar growth on both SD-Leu-Ura and SG-Leu-Ura plates (Fig. 2). Thus, the GPI anchor is not essential for PrP suppression of Bax-induced death. Western blots confirmed that DPAPB-PrP230 is glycosylated, indicating entry into the secretory pathway, and that it does not affect expression of Bax (supplemental Fig. S3*A*).

Deletion of Residues 23–31, but Not the Octapeptide Repeats, Reduces Rescue Activity—To further map structural determinants in PrP that are essential for suppressing Bax-induced cell death, we generated a set of deletions covering the unstructured, N-terminal region of the protein. DPAPB-PrP molecules carrying several different deletions that lie between residues 32 and 93 (Δ51–90, Δ32–80, Δ32–93) were as active as wild-type DPAPB-PrP254 in rescuing growth on galactose plates

(Fig. 3). Thus, the octapeptide repeats (residues 51–90) are not essential for activity. Interestingly, however, deletion of only 9 residues within a positively charged region following the signal peptide (DPAPB-PrPΔ23–31) significantly reduced rescue activity (Fig. 3). Colony counts on glucose and galactose plates (performed as in Fig. 1*B*) indicated that DPAPB-PrPΔ23–31 has about 15% of the activity of wild-type PrP. All of the deleted proteins were expressed at levels similar to that of wild-type DPAPB-PrP254, and all were glycosylated, as assessed by Western blotting (supplemental Fig. S3*B*). In addition, all strains expressed similar levels of Bax when grown on galactose (supplemental Fig. S3*B*).

PrP Molecules Carrying an Octapeptide Insertion, but Not One of Several Point Mutations, Fail to Suppress Bax-mediated Cell Death—We tested DPAPB-PrP molecules carrying either a point mutation (E199K, P101L, D177N, F197S) or a nine-octapeptide insertional mutation (PG14) whose human homologues are associated with inherited prion diseases (27). None of the mutants impaired the ability of yeast to grow on glucose plates, implying that these proteins were not toxic in the absence of Bax (Fig. 4*A*). However, the PG14 insertion abolished growth on galactose plates, implying that this mutation abrogated the rescue activity of PrP. In contrast, the point mutants displayed full rescue activity, as evidenced by unimpaired growth on galactose plates. Western blots confirmed that PrP and Bax were expressed in each of these strains after galactose induction (supplemental Fig. S3*C*).

The cytoprotective activity of the mutant proteins correlated with their biochemical properties. We measured detergent insolubility and protease resistance, two biochemical attributes of PrP^{Sc}. Only PG14 PrP exhibited protease resistance, yielding a 27–30-kDa protected fragment, while the point mutants were completely digested under the same conditions (Fig. 4*B*). In addition, a substantial amount of the glycosylated form of PG14 PrP was insoluble, while the glycosylated forms of the point mutants were mostly soluble (Fig. 4*C*). The unglycosylated forms of all PrP molecules, including wild-type, were insoluble, as is the case when these proteins are expressed in mammalian cells (28). Thus, the lack of Bax rescue activity of PG14 PrP correlates with its PrP^{Sc}-like biochemical properties.

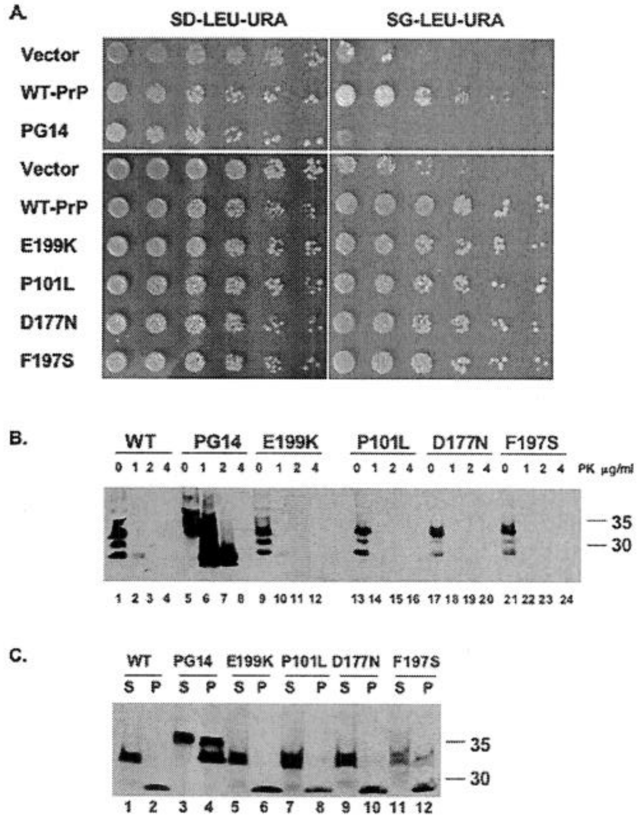

FIG. 4. **PrP molecules carrying an octapeptide insertion, but not one of several point mutations, fail to suppress Bax-mediated cell death.** Yeast expressing Bax under control of a galactose-inducible promoter were transformed with empty p426GPD vector (*Vector*) or with p426GPD vector encoding DPAPB-PrP254 (*WT-PrP*) or DPAPB-PrP254 carrying the indicated mutations (PG14 (9-octapeptide insertion), E199K, P101L, D177N, F197S). Serial dilutions of cultures were spotted onto SD-Leu-Ura (glucose) or SG-Leu-Ura (galactose) plates as described in the legend to Fig. 1. *B*, yeast expressing the indicated proteins were grown to log phase in SD-Leu-Ura liquid medium and were then transferred to SG-Leu-Ura medium for 18 h to induce Bax expression. Cell lysates were digested with the indicated amounts of PK, after which PrP was visualized by Western blotting using 3F4 antibody. *Lanes 1, 5, 9, 13, 17*, and *21* represent 200 μg of protein and the other lanes 800 μg of protein. The diglycosylated, monoglycosylated, and unglycosylated forms of PG14 PrP, which are not well separated in *lane 5*, migrate at 37, 35, and 33 kDa, respectively. *C*, yeast were grown as described for *B*. Cell lysates were centrifuged at 186,000 × *g* for 45 min, after which PrP in supernatant (*S*) and pellet (*P*) fractions was analyzed by Western blotting using 3F4 antibody.

DISCUSSION

In this study, we have demonstrated that PrP targeted to the secretory pathway of *S. cerevisiae* is capable of rescuing the lethal phenotype caused by heterologous expression of Bax, a pro-apoptotic member of the *Bcl-2* gene family. In mammalian cells, Bax is a cytoplasmic protein that is translocated to mitochondria in response to apoptotic signals, where it promotes cell death by mediating release of cytochrome *c*, which in turn activates caspase-dependent pathways (29). Although *S. cerevisiae* does not contain endogenous *Bcl-2* family members or caspases, the initial events underlying Bax activity in yeast and mammalian cells are similar, including translocation of the protein to mitochondria, release of cytochrome *c*, and alterations in mitochondrial function (17, 30). The downstream effectors of Bax-induced cell death in yeast are less certain, but may include reactive oxygen species generated in mitochondria. Of note, several mammalian proteins that were isolated by virtue of their ability to suppress Bax-induced cell death in yeast also have Bax inhibitory activity in mammalian cells (18–20). Thus, it is likely that the ability of PrP to rescue the lethal phenotype of Bax-expressing yeast reflects interaction with Bax-related pathways that are operative in higher organisms.

How does PrP protect yeast from Bax-induced cell death? One possibility is that PrP directly associates with and inactivates Bax. Although PrP was reported to interact with Bcl-2 in a yeast two-hybrid assay (31, 32), binding of DPAPB-PrP to Bax in our expression system seems unlikely, since DPAPB-PrP is present in the secretory pathway while Bax is in the cytoplasm. Indeed, when we deliberately targeted PrP to the cytoplasm by deletion of the N- and C-terminal signal sequences (PrP23–231), the protein lost its Bax rescuing activity. Whether the inactivity of cytosolic PrP is due to protein aggregation or to other factors remains to be determined. A second, more likely possibility to explain the protective effect of PrP is that PrP interacts with endogenous yeast proteins that lie downstream of Bax in a cellular stress or toxicity pathway. In addition to members of the *Bcl-2* family, several other mammalian proteins are known to inhibit the action of Bax (18–20), and it is possible that yeast homologues of these or related molecules play a role in the PrP rescue pathway we have demonstrated here.

The results reported here are relevant to theories about the pathogenicity of mutant PrP molecules associated with inherited prion diseases. One hypothesis is that these molecules acquire toxic properties by virtue of misfolding or being converted to a PrP^{Sc}-like state. Alternatively, the presence of the mutation may abrogate or alter a normal physiological function of PrP^{C}. We found that PrP molecules carrying a nine-octapeptide insertion (PG14), but not one of several point mutations, failed to suppress Bax-mediated cell death. In addition, PG14 PrP was the only one of the mutants that showed significant protease resistance and detergent insolubility when expressed in yeast. These results suggest that loss of Bax-protective function, perhaps as a result of protein aggregation or misfolding, could play some role in PG14-associated prion disease. This conclusion is consistent with our observation that neuronal death in Tg(PG14) mice is Bax-dependent (33). It is possible that this mechanism is also applicable to other pathogenic mutants, which display PrP^{Sc}-like biochemical properties in cultured mammalian cells (34) and brain (27), even though they do not in yeast.

PrP has also been reported to protect against cell death in several mammalian systems. The studies that are most directly comparable to ours are those in which human PrP was shown to prevent Bax-induced apoptosis of cultured human neurons, following microinjection of plasmids encoding these proteins (9, 12). In those studies, in contrast to ours, PrP containing the D178N mutation (equivalent to D177N in mouse) was reported to lack rescuing activity, while cytoplasmic PrP-(23–231) retained activity. On the other hand, PrP lacking the GPI anchor was active in both systems. PrPΔ56–88, which retains part of the first octapeptide repeat, was inactive in neurons, but we did not test this construct in yeast. It is possible that the discrepancies between yeast and human neurons reflect fundamental mechanistic differences in how PrP suppresses Bax-induced cell death in the two systems. Alternatively, the pathways involved in PrP cytoprotection may be partially, but not completely, conserved between yeast and mammals. Interestingly, PrPΔ32–93, which rescues Bax-induced cell death in yeast, is also capable of rescuing neurodegeneration in transgenic mice expressing PrPΔ32–134 (15). Moreover, deletion of a charged region encompassing residues 23–31 partially abolishes PrP rescue activity in yeast, and a slightly smaller deletion within the same region (Δ23–28) has been found to abrogate protection against Doppel-induced apoptosis in mouse neurons (35). Taken together, these comparisons suggest that the ability of PrP to suppress Bax lethality in yeast reflects a physiological activity of PrP that is operative in mammals and that may protect cells against several kinds of toxic insults.

The observations reported in this paper establish yeast as a valuable tool to assay a potentially important biological activity of PrP. This system offers many advantages, most importantly, the capability of genetic analysis of PrP function. With the use of appropriately designed genetic screens and selections, it should now be possible to identify proteins from yeast and mammals that alter the cytoprotective activity of PrP and that may also interact physically with PrP.

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